Precise domain specification in the developing Drosophila embryo

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A simple morphogen gradient based on the protein *bicoid* is insufficient to explain the precise (*i.e.* similar in all embryos) setting of antero-posterior gene expression domains in the early *Drosophila* embryo. We present here an alternative model, based on quantitative data, which account for all of our observations. The model also explain the robustness of hunchback(*hb*) boundary setting in unnatural environments such as published recently [Luccheta et al., Nature, 434:1134-8(2005)]. The model is based on the existence of a secondary gradient correlated to *bcd* through protein degradation by the same agent.

I. INTRODUCTION.

The specification of gene expression domain in a developing embryo is the central problem of developmental biology. For *Drosophila Melanogaster*, the early events for the antero-posterior differentiation are well known : bicoid (bcd) mRNAs are deposited and localized by the mother at the anterior pole of the embryo. This localised source serves as a "fountain" for bcd proteins, which diffuse from there and are degraded throughout the embryo by some protease. The combined processes of diffusion from a source and degradation generate a stable, stationary (time independent) gradient of the bicoid protein with high concentration at the anterior and low concentration at the posterior pole . Downstream genes *read* the *bcd* concentration and set their boundaries according to different thresholds. The readout process can be direct, as in the case of hunchback or indirect, as for the gap genes which are activated by reading a combination of bicoid and hunchback (and each others). This is a cascading gene interaction network where *bcd* and *hb*, at the top, play a very special role. The general scheme for this kind of genetic network is called (simple) "morphogen gradient model"[1] (Fig.1(a-c)).

This picture has emerged over the past twenty years following several fundamental discoveries: (i) Bcd is a maternally active gene that encodes a transcription factor for many downstream genes expressed in the early embryo; (ii) bcd mRNAs are localised at the anterior pole of the embryo ; (iii) *bcd* protein concentration profile forms a gradient across the embryo ; (iv) modifying bcd dosage shifts downstream gene expression domains, in "agreement" with a threshold reading process[2, 3] (Fig.1(d)).

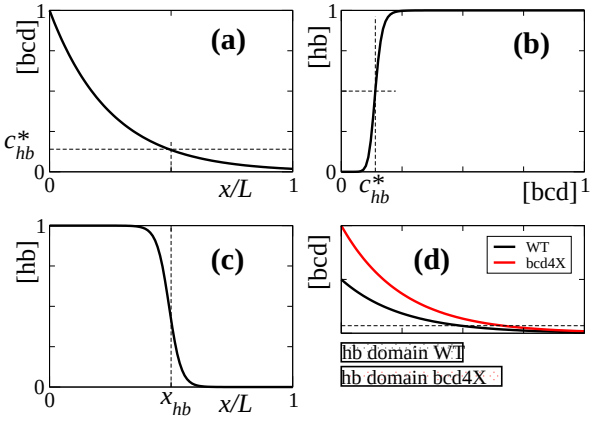

Figure 1: "Color online" (a-c) : Outline of the morphogen gradient model. (a) The concentration of a transcription factor protein (such as bcd) varies across the embryo (of size L) ; in this sketch, we suppose an exponential variation : $[\mathrm{bcd}] = \exp(-x/\lambda)$. (b) The transcription factor activates a downstream gene (such as hb) according to a Hill law $[\mathrm{hb}] = [\mathrm{bcd}]^n/\{(c^*_{hb})^n + [\mathrm{bcd}]^n\}$. The Hill coefficient n is supposed high enough for the activation to be switch like : for bcd concentration below the treshold concentration c^*_{hb}, there is little hb production ; hb production on the other hand is at its maximum when $[\mathrm{bcd}] \gtrsim c^*_{hb}$. (c) The hb gene is activated (and hb protein produced) only in the part of the embryo where $[\mathrm{bcd}] > c^*_{hb}$. Here, only cells whose position $x < x_{hb}$ transcribe the hb gene where x_{hb}, the boundary of hb domain, is given by $x_{hb} = -\lambda \log c^*_{hb}$. (d) Dosage modification experiment : if the amplitude of the morphogen gradient is doubled (compared to wild type), the position at which the gradient crosses a given threshold is pushed further upward. In this example, hb expression domain boundary (boxes) would be shifted by $\lambda \log 2$.

This simple morphogen gradient model suffers however from two important weaknesses : precision and scaling[4]. Precision here is to be understood as the degree of similarity between gene expression domains in different embryos. Scaling signifies the proportionality between gene expression domains and the embryo size.

Precision. The *bcd* concentration profile depends on various parameters such as the diffusion coefficient, *bcd* half life -which itself depends on the degrading agent concentration- and the quantity of mRNA deposited by the mother at the pole. Any embryo to embryo variation in these parameters will modify the *bcd* profile and thus affect the spatial extension of domains in these embryos. The problem with the simple morphogen gradient model is the lack of feedbacks and error correcting mechanisms. No usable machine however can be made without feedbacks.

Scaling. The shape of the *bcd* gradient is set by the above cited parameters, and none of them depends on the size of the embryo. This means that even if the mother were able to control precisely these parameters, the spatial extension of the *hb* domain will not be proportional to the *embryo size*.

As an example, if HB is activated at a Bcd concentration threshold normally found at 230 microns, that concentration will occur at the same average distance from the anterior end of the egg regardless of its length. In a 450 μm long embryo, *hb* domain will represent 51% embryo length (EL), whereas in a 500 μm embryo it will occupy only 46% EL. This "error" is equivalent to five nuclei. Any mechanism used to create the scaling has to sense the posterior end of the egg, and this is clearly lacking in a simple morphogen gradient. Of course, in the Drosophila embryo, there are known posterior gradients, such as *nanos* which participates in the boundary setting of *hb*, and *caudal*, but we know that these genes do not have any role in the scaling of *hb*[4].

In our previous investigation [4], we have shown that indeed, *bcd* is an extremely noisy signal and varies widely from embryo to embryo. The positional information *bcd* can transfer to hb, based solely on a threshold reading mechanism, has a standard deviation of 7% EL (approximately 7 nuclei). If the human nose were positioned by such a morphogen, we would find it in some individuals on the torso, and in some on top of the head ! Moreover, there is no correlation between the positional information of *bcd* and the egg size. Hb on the other hand displays astonishing precision and scaling: at cycle 14, its boundary is set at 0.49 ± 0.01 EL, always proportional to the embryo size. In fact, *hb* plays the role of the "real" morphogen[5], filtering out all the errors of *bcd* and transmitting a pure signal to downstream genes. Boundaries of downstream genes show strong correlation to *hb* fluctuations, whether in WT embryos or mutants where *hb* itself looses its precision[6]; on the other hand, no correlation with *bcd* fluctuation can be observed. We use the term *fluctuation* or *noise* through this article as an equivalent of "embryo to embryo variability".

Clearly, there are correcting mechanisms present in the developing embryo which compensate for *bcd* errors at the very first stage of boundary setting. There are two possibilities for such a mechanism, either zygotic or maternal. Zygotic models are based on the subsequent interactions between hb and other gap genes such as Kr, kni and gt. A look at the genetic network of antero-posterior early drosophila differentiation shows that indeed many such feedbacks are thought to exist[7]. Some of them, upon closer inspection, are wrong (Kr)[6], but others do have a noticeable effect on the mean position of the *hb* boundary. No zygotic gene however has an effect on the *precision* and *scaling* properties of *hb* . Even removing 80% of the Drosophila genome had no visible effect on hunchback [4]. Another counter argument for zygotic feedback is the timing of the events : hb is among the very first activated genes, and from the very beginning, it displays high precision.

The other possibility is a maternal control : if the mother provides another signal to the embryo, and if this second signal has the same source of fluctuation as that of *bcd* (*i.e.* the two signals are correlated), then in principle the two noises can cancel out each other. We have previously shown that *nanos*, or more generally, genes downstream of oscar, do not play the role of this secondary signal. Some alleles of the maternal gene stauffen however disrupt the precision of the *hb* boundary, inducing fluctuation of the same magnitude as that of *bcd*. *Sta* itself is not a transcription factor, but plays a role during oogenesis in the localization of anterior *and* posterior mRNAs[8].

In the reminder of this article, we will first investigate certain aspects of the *bcd* gradient. We will then explore the error correcting capabilities of a (hypothetical) secondary signal. We will show that such a model is in extremely good agreement with ours and others observations.

II. THE *BCD* GRADIENT.

Let us revisit the establishment of the bicoid gradient. As mentioned above, *bcd* proteins are produced at the anterior pole at a rate J, diffuse through the embryo with a diffusion coefficient D, and are degraded by some agent at a rate ω. The concentration $B(x,t)$ of *bcd* is given by the diffusion (Fick's) equation $\partial_t B = D\partial_x^2 B - \omega B$ with the boundary conditions $\partial_x B|_{x=0} = -J$ and $\partial_x B|_{x=L} = 0$. L is the embryo length. The second boundary condition expresses the fact that *bcd* molecules cannot cross the posterior extremity of the embryo. After a transitory time a stable, stationary ($\partial B/\partial t = 0$) state is reached which obeys

$$\frac{d^2B}{dx^2} - \frac{1}{\lambda^2}B = 0 \tag{1}$$

where $\lambda = \sqrt{D/\omega}$ is the diffusion length, *i.e.* the average distance a molecule diffuses before degradation. The stationary solution reads:

$$B(x) = C_1 \exp(-x/\lambda) + C_2 \exp(+x/\lambda)$$

The amplitudes are $C_1 = J\lambda/(1 - \exp(-2L/\lambda))$ and $C_2 = \exp(-2L/\lambda)C_1$. For *bcd* gradient in Drosophila, the average diffusion length is $\lambda = 0.26L$. We can thus drop the positive exponential and approximate the gradient by

$$B(x) \approx (J\lambda)\exp(-x/\lambda)$$

The error in the approximation is 0.03% at the anterior and 2% at the posterior pole.

The measurement of the *bcd* gradient is most conveniently achieved by immuno-fluorescence staining techniques : in each embryo the local intensity of fluorescence

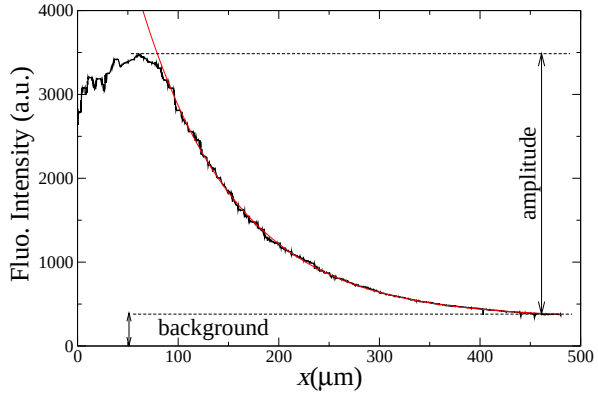

Figure 2: "Color online" The profile of the *bcd* gradient in a bcd6X embryo measured by immuno-fluorescence. The fluorescence intensity data (black) is fitted to an exponential (red) $a\exp(-x/\lambda)+b$.

$I(x)$ staining is extracted by image analysis techniques (see Material and Methods). Figure 2 displays such a measurement in one embryo.

In order to assess embryo to embryo variability, the most robust parameter to measure is the diffusion length λ, which is independent of the fluorescent staining noise and can be obtained by directly fitting the function $a\exp(-x/\lambda)+b$ to $I(x)$. As reported before, the embryo to embryo variability of the diffusion length is $\delta\lambda = 0.05$ EL. It follows that if Hb was activated directly by *bcd*, the embryo to embryo variability of its boundary would be $\delta x_{hb} = 0.07$ (approximately seven nuclei).

There exists a possibility for this signal to be less noisy than it appears, at least for Hb activation at mid-embryo : If in an embryo, the *bcd*-degrading agent has a higher concentration, then λ is smaller and a smaller *proportion* of molecules produced at the anterior pole reach the mid embryo ; if however, in the same embryo, more Bcd mRNA is deposited at the pole, the synthesis rate at the origin would be higher. The combination of these two effects can induce the same *number* of *bcd* molecules to reach the middle of the embryo. Individual absolute amplitudes (Fig.2) cannot be measured directly from the images, because of the additional uncertainty induced by fluorescent staining, but if the above hypothesis were true, we should observe a negative *correlation* between the amplitude a of the fitted signal and its slope λ. No significant correlation has been found however : on $N = 91$ samples analysed, the spearman correlation coefficient obtained was $r_{sp} = -0.09$ ($P = 0.4$).

As we mentioned above, individual signal amplitudes cannot be measured for embryos. It is possible however to compare the *average* signal amplitudes in different backgrounds if embryos are stained at the same time in the same conditions. Then, the experimental error induced by staining is similar for all embryos and by averaging the amplitudes over enough embryos in the same background, a good estimation of the signal strength in one background *compared* to the other can be obtained (see Material and Methods).

Background	Rel. Ampl.	N
Bcd2X(WT)	1.0 ± 0.1	16
Bcd4X	1.6 ± 0.1	17
Bcd6X	1.9 ± 0.1	21

Table I: Average Relative (to WT) amplitude of the *bcd* gradient in embryos derived from mothers with variable numbers of Bcd transgenes. Uncertainties are standard errors (standard deviation/$\sqrt{N}$). The relative amplitude is approximately $RA = 2\times0.5+n\times0.25$ where 2 is the number of WT copies of bcd, n the number of transgenes, 0.5 the efficiency of a WT gene and 0.25 the efficiency of a transgene.

Temp.	Rel. Ampl.	λ	N
29C	0.80 ± 0.03	0.28	32
25C	1.0 ± 0.04	0.26	19
18C	0.84 ± 0.03	0.37	24
9C	0.68 ± 0.06	0.80	15

Table II: Average *bcd* gradient amplitude at various temperature, and its exponential decay length (%EL). Amplitudes are relative to 25C condition.

Table I shows the *bcd* average amplitude in Wild type embryos and in embryos derived from mothers with 2 or 4 more copies of Bcd (see Materials and Methods). There is *a priori* no reason for a transgene to have the same efficiency than the endogenous locus as observed here : each transgene addition increases the total transcription rate by approximately half of endogenous expression.

Table II displays the *bcd* amplitude in embryos developing at different temperature. *Bcd* amplitude decreases and its diffusion length increases as the temperature drops below 25C, implying a slowing down of both protein synthesis and degradation.

III. SECOND MORPHOGEN HYPOTHESIS.

In the simplest model of antero-posterior specification, Hb is activated when the *bcd* signal is above a given threshold c_0. In this model, *hb* boundary would be as (embryo to embryo) variable as *bcd*. As we mentioned above, the *bcd* error can be corrected maternally if a second morphogen were present in the embryo and its fluctuations correlated to that of *bcd*. Then, the errors of these two signals can cancel each other. Let us again insist that even though nanos participates in the *hb* boundary setting (indirectly, by degrading maternal hb mRNA), it is *not* the second morphogen considered here : its removal (with or without the maternal hb) does not affect the precision of *hb*.

The *bcd* variability we measure is in fact the variability of its exponential decay length λ (which has a standard deviation of 0.05 EL). The decay length in turn depends on the diffusion coefficient D of *bcd* molecules and the

degradation rate ω. The diffusion coefficient, a passive parameter which is related to viscosity should not vary from embryo to embryo. On the other hand, the degradation rate depends on the quantity of degrading agents the mother deposit in the embryo which can be highly variable and the main source of fluctuations in *bcd* gradient. In order for the second morphogen to be correlated to *bcd*, it will be enough for it to be degraded by the same agent which degrades *bcd*. Then, in a given embryo, if ω is higher (lower) than average, both morphogens will have a smaller (bigger) diffusion length.

The other fact we mentioned is the scaling property of *hb* boundary. Whatever the activation mechanism, it has to be influenced by the distance to both poles[9]. *Bcd* has a vanishing value at the posterior pole and cannot play such a role.

The simplest model of a second morphogen (which we will designate by the letter *P)* taking into account the above ideas is the following : (i) P is produced from a localized source of mRNA at the *posterior* pole, diffuses and is degraded with the same rates as *bcd*, so it makes an exponential gradient going from high values at the posterior to small values at the anterior ; (ii) it is an *inhibitor* of Hb. More precisely, Hb is activated in the region where $B(x) > P(x)$, so the boundary position of *hb* is given by the condition (Fig. 3) :

$$B(x_{hb}) = P(x_{hb}) \tag{2}$$

This condition is easily realised for example if *bcd* and P compete for the same sites on the regulatory region of the Hb gene. In principle, we should speak about the activities of these two proteins and not their actual concentration. P can have twice the activity of *bcd* and be present at half the concentration. Without loss of generality however, and to keep the model as simple as possible, we will use concentrations instead of activities.

Repeating the arguments of the introduction, $P(x)$ obeys the diffusion equation $d^2P/dx^2 - \lambda^{-2}P = 0$ with the boundary conditions $dP/dx|_{x=0} = 0$ and $dP/dx|_{x=L} = J$ (production at the posterior pole). Thus $P(x)$ reads (Fig. 3)

$$P(x) \approx (J\lambda)\exp\left[(x-L)/\lambda\right] \tag{3}$$

where in *each embryo*, the diffusion length for both morphogens are the same. This hypothesis is a consequence of P being degraded by the same agent as *bcd*. Let us now consider the plausibility of this model and its many prediction.

Correcting for errors and scaling. If Hb was activated only by *bcd* through a thresholding mechanism $B(x_{hb}) = c_0$, its embryo to embryo fluctuation would be $\delta x_{hb} = -\delta\lambda \ln c_0 = 0.07$(in EL units). If however Hb was activated by two gradients as explained above, the condition (2) reads

$$\exp(-x_{hb}/\lambda) = \exp\left[(x_{hb}-L)/\lambda\right]$$

and thus the *hb* boundary is given by (see Fig. 3)

$$x_{hb} = L/2$$

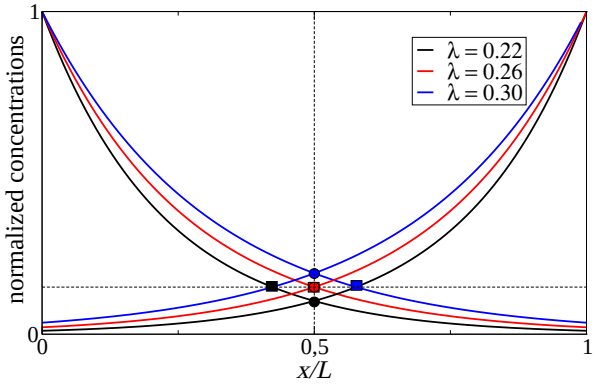

Figure 3: "Color online" Setting of *hb* boundary in a two gradients model (circles) and a simple gradient model (rectangles). 3 couples of anterior ($B(x)$) and posterior ($P(x)$) gradient (normalized to $[0,1]$) with diffusion length $\lambda = 0.22, 0.26, 0.30$ are shown. In a two gradient model, the position of *hb* boundary, given by the condition $B(x_{hb}) = P(x_{hb})$, marked by circles, does not vary and remains at $L/2$. Compare to a simple gradient model where the *hb* boundary is specified by the anterior gradient crossing a given threshold, marked by rectangles ; the *hb* position would follow variations of λ.

We have here two remarkable facts. First, the position of *hb* is set independently of λ, the source of fluctuations : The errors in the two gradients have cancelled out each other. Second, the *hb* position is automatically proportional to the embryo size L. This simple model explains two of the elusive behaviors of Hb activation.

Let us also note that by this mechanism, when both posterior and anterior gradient have the same slope, error correction is optimal at mid-embryo, precisely where *hb* boundary is actually set. In principle, the two gradients mechanism can set the boundary at any position if the condition for the gene activation were $uB(x_{hb}) = P(x_{hb})$. Then, the position of *hb* boundary would be

$$x_{hb} = \lambda(\ln u)/2 + L/2$$

But this would not be proportional to the embryo size L. Even worst, fluctuations in λ would not be corrected any more and

$$\delta x_{hb} = \delta\lambda\,(\ln u)/2 \tag{4}$$

In the general case, anterior and posterior gradients can have different slope, and the position of a given gene's boundary will be given by $u\exp(-x/\lambda_1) = \exp((x-L)/\lambda_2)$, *i.e.*

$$x = \frac{\lambda_1}{\lambda_1+\lambda_2}(L + \lambda_2 \ln u)$$

Again, scaling can be achieved only if $u = 1$. Moreover, If we suppose the embryo to embryo fluctuations of slopes $\delta\lambda_i$ to be due to the degrading agent concentration fluctuations δC and thus $\delta\lambda_i/\lambda_i = (1/2)\delta C/C$, then

$$\delta x = \left[(\lambda_1 \ln u)/(\lambda_1+\lambda_2)\right]\delta\lambda_2$$

Background	u	measured	Simple grad.	Two grads
Bcd1X	0.5	-0.08 ± 0.01	-0.18	-0.09
Bcd4X	1.6	0.07 ± 0.015	0.12	0.06
Bcd6X	1.9	0.10 ± 0.02	0.17	0.08

Table III: Measured shift in *hb* boundary (relative to its position in WT) compared to predictions of simple and two gradients model. The quantity u denotes the strength of *bcd* amplitude in different genetic background. *Bcd* amplitudes in mothers with transgenes have been measured (table I).

and the error is corrected again only if $u = 1$.

A last issue is error correction for amplitude variations. There exists *a priori* an embryo to embryo variation in the quantity of mRNA deposited by the mother which we have neglected in the above discussion. This source of fluctuation - which cannot be measured by fluorescent staining and is independent of variation in diffusion length - would add to errors in *hb* boundary. This error also can be corrected by the two gradients mechanism, if there is correlation between mRNAs of localized posterior and anterior morphogens, *i.e.* the same quantity is deposited at both poles. It is probably no coincidence that the only gene which disrupts the precision of *hb* is one that is responsible for mRNA localisation at *both* poles.

Effect of bcd gene copies. The most convincing argument for bicoid being indeed a morphogen was given by Driever and Nusslein-Wolhard in 1988 when they showed that providing more or less copies of Bcd gene to the embryo by the mother shifts the position of downstream genes toward anterior or posterior in the embryo[3]. This argument however does not hold quantitatively if *bcd* were the sole morphogen. By modifying the number of genes in the mother, the amplitude of *bcd* in derived embryos becomes u times higher than in WT ($u = 1$ for WT). Then the condition $B(x_{hb}) = u \exp(-x/\lambda) = c_0$ of the simple morphogen gradient model implies that the expected shift (compared to WT) in average *hb* position be $\Delta x_{hb} = \lambda \ln u$. In a two gradients model however, the expected shift in *hb* boundary will be only half of this value : $\Delta x_{hb} = (\lambda/2) \ln u$ (Fig.4). Table III shows the comparison between measured shifts in embryos with various background and values expected from simple and two gradients model. As it can be observed, only the two gradients model is in agreement with the measurement. Note that if we had assumed a normal activity for transgenes ($u = 2$ for bcd4X and $u = 3$ for bcd6X), the discrepancy of the simple morphogen model would be much higher, but the two gradients model predictions will still be acceptable.

Temperature compensation. As showed in table II *bcd* amplitude and diffusion length are function of temperature. There is no reason *a priori* for a simple morphogen gradient to be temperature compensated, *i.e.* specify the same boundary position for *hb* whatever the temperature. Without more knowledge of the detailed activation rates however, this possibility cannot be ruled out, at least

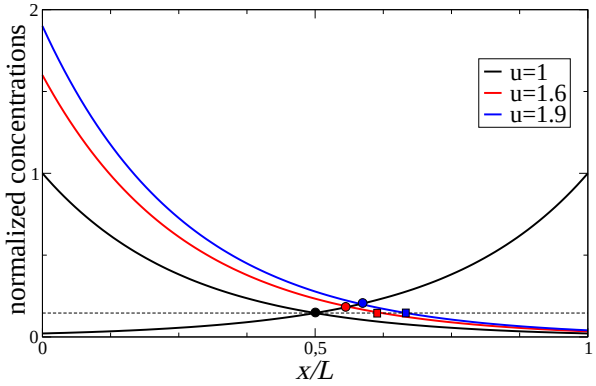

Figure 4: "Color online" *Hb* boundary shifts when the strength u of the anterior gradient (but not of the posterior gradient) is varied. The shift expected from a two gradients model (circles) is half of a simple gradient model (rectangles). Concentrations (y axis) are relative to WT.

for the activation of a single gene : the Hb activation threshold can vary in such a manner as to compensate the variation in the other two parameters. This however remains a fragile process with no feed back. A two gradients mechanism on the other hand corrects naturally for temperature variations : if for example *bcd* and P compete for the same regulatory region, they are affected in a similar way by temperature variation and the condition $B(x_{hb}) = P(x_{hb})$ remains valid at all temperatures.

Precision in a non-uniform temperature gradient. Using a microfuidics device, Luccheta *et al.* have been able to keep one half of an embryo at one temperature (18C) and the other half at another temperature (25C)[10]. Even though development time is highly different in the two halves, the *hb* boundary is still set at mid-embryo with high precision. As we will show below, This is what a two gradients model predicts. The nature of this regulation is summarized in Fig.5 , where the posterior half is maintained at 18C and the anterior half at 25C. The source (mRNAs) for the posterior gradient being at 18C, synthesis of P is reduced at the posterior pole. The lower temperature in the posterior half of the embryo however induce also an increase in the diffusion length in this part. As a consequence the *number* of P molecules reaching the mid-embryo are the same that the number of *bcd* molecule coming from the anterior pole.

More precisely, a variation in temperature affects the synthesis rate J and the diffusion length λ for which we possess quantitative data. The diffusion equation for *bcd* reads in this non-uniform temperature gradient

$$\frac{d^2B}{dx^2} - \frac{1}{\lambda(x)} B(x) = 0 \qquad (5)$$

with $\lambda(x) = \lambda_1$ if $x < L/2$ and $\lambda(x) = \lambda_2$ if $x > L/2$, where subscript 1 and 2 refer to diffusion length in the anterior and posterior halves of the embryo, set by temperature in these parts. Two of the boundary conditions read as before $d^2B/dx^2|_{x=0} = -J_1$ and $d^2B/dx^2|_{x=L} = 0$. J_1

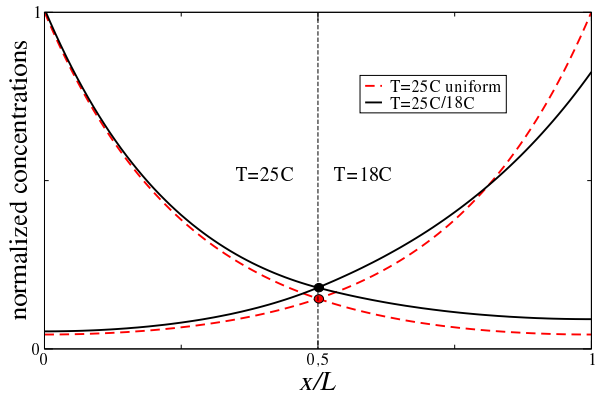

Figure 5: "Color online" Anterior and posterior gradients for the setting of *hb* boundary in a non-uniform temperature gradient. The anterior half of the embryo is maintained at 25C and its posterior half at 18. Values for synthesis rates and diffusion lengths are set accordingly to Table II. The position $B(x_{hb}) = P(x_{hb})$ is marked by a circle. For comparison, the two gradients are also shown in a uniform temperature field. Concentrations ($y$axis) are relative to uniform temperature 25C.

is the synthesis rate of *bcd* at the anterior pole set by local temperature there. There are two additional conditions of continuity of concentration and flux at the boundary between high and low temperature : $B(L/2^-) = B(L/2^+)$ and $dB/dx|_{x=L/2^-} = dB/dx|_{x=L/2^+}$. The solution for the gradient is now two connected exponentials

$$B(x) = C_1 \exp(-x/\lambda_1) + D_1 \exp\left[(x-L)/\lambda_1\right] \qquad x \le L/2 \quad (6)$$
$$= C_2 \exp(-x/\lambda_2) + D_2 \exp\left[(x-L)/\lambda_2\right] \qquad x > L/2 \quad (7)$$

The four amplitudes C_i, D_i are determined by the linear system of four equations given by boundary conditions. The same equation (5) holds for the gradient $P(x)$ except that two of the boundary conditions are reversed: $d^2P/dx^2|_{x=0} = 0$ and $d^2P/dx^2|_{x=L} = J_2$. Note that J_2 is the synthesis rate at the posterior pole, set by local temperature there.

Figure 5 shows the solutions of the above equations when the anterior half of the embryo is held at 25C and its posterior half at 18C, similar to the experiment performed by Lucchetta *et al.*([10],Fig4a). All values for diffusion lengths and synthesis rates are measured experimentally (table II). As it can be observed, the position of *hb* boundary x_{hb} given by the condition $B(x_{hb}) = P(x_{hb})$ is equal to its value for an embryo in a uniform temperature field. Moreover, precision is still conserved and variations of 5% in the diffusion lengths ($\delta\lambda = 0.05$) induce only 10 times smaller variations in *hb* boundary ($\delta x_{hb} = 0.005$).

IV. CONCLUSION.

The simple morphogen model, where *bcd,* in a concentration dependent manner, specifies gene expression domains lacks feedback mechanisms and cannot quantitatively account for many phenomena. These phenomena include high precision and scaling properties of downstream genes; smaller than expected shift when the amplitude of *bcd* is changed; temperature compensation, specially when embryos are maintained in non-uniform temperature field.

We have shown in this article that all these phenomena can be accounted for if we suppose the existence of a second posterior morphogen correlated to *bcd*. The correction mechanism in this "two gradients model" is based on a simple principle : if a signal is noisy, duplicate it by taking its mirror image and subtract the second from the first. Then, at one position inside the embryo (the mid-embryo being the optimum choice), the noises of the two gradients cancel each other completely. This is where the *hb* boundary is set and this precise signal can then be transmitted to downstream genes. It is remarkable how such a simple model can explain so many different observations, either obtained by us or recently by Luccheta *et al.*

The second gradient remains however a hypothesis and it would seem surprising that more than 20 years after the genome wide screen there are still genes not uncovered. Until (and if) the second morphogen is found, the "two gradients" model is only a plausible framework, similar to the "simple gradient" model until 1987 and the discovery of bcd. It is however significant that the only mutation we have found which disrupt the precision of boundary setting is the *maternal* gene Stauffen, which is responsible for localizing mRNAs at both anterior and posterior poles. More work is needed at this level to understand the nature of molecular events caused by Stauffen mutation. We believe however that the exact mechanism cannot be very different from the general scheme we have presented here.

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Appendix A: Material and Methods

1. Staining and Image analysis.

Embryos were collected at cycle 14 and immunostained following published protocols[11], except for the final rinsing time which is an important step in reducing non-specific antibodies attachment. The best results were obtained by 3 days rinsing (signal to noise ratio of ≈ 20 can be obtained). Most results were obtained by 1 day rinsing (S/N$\approx$ 10). When studying temperature effects, embryos were collected for 1h at 25C and then allowed to reach cycle 14 at the set temperature (20h at 9C). Antibodies were a gift of J. Reinitz and David Kossman[12]. High resolution (1317×1015pixels, 12bits/pixel) images of stained embryos in a given condition were taken. Images were focused at mid-embryo to avoid geometric dis-

tortions. Intensity profiles were extracted by sliding a rectangle, the size of a nucleus, along the inner edge of the embryo, itself detected by intensity thresholding. The average was computed on the brightest half pixels of the rectangle in order to compensate for the space between nuclei. The coordinates of the rectangle were projected on the main axes of the embryo, and the intensity $I(x)$ recorded separately for dorsal and ventral side.

2. Amplitude quantification.

The bcd concentration $B_i(x)$ in the embryos i reaches a high value at x_i^* which we call its *amplitude* A_i ($A_i = B_i(x^*)$) (Fig. 2) and drops to vanishing level at the posterior pole $B_i(L) \approx 0$. In the fluorescent signal we measure in this embryo, a part β_i is due to non-specific antibody binding and another part α_i to specific ones. Therefore, the fluorescent intensity in this embryo reads

$$I_i(x) = \alpha_i B_i(x) + \beta_i$$

α, β and $B(x)$ are random variables (varying from embryo to embryo) with given averages and standard deviations. If all embryos from various background are stained at the same time in the same conditions, then α and β have the same distribution for all embryos and depend only on staining conditions. $B(x)$ on the other hand depends only on the genetic background of the embryos. Using the exponential variation of *bcd* in each embryo, the term β_i can be evaluated as $I_i(L)$. Therefore the *measured* quantity we call "signal" $S_i = I_i(x^*) - I_i(L) = \alpha_i A_i$ depends only on two random variables. Averaging over all embryos in a given background, and assuming independence of α and A :

$$\langle S \rangle_{\text{bckgrnd1}} = \langle \alpha \rangle \langle A \rangle_{\text{bckgrnd1}} \tag{A1}$$

To compare the relative amplitude of *bcd* in two different backgrounds, one has only to evaluate the ratio of the average signals in these two backgrounds:

$$\frac{\langle A \rangle_{\text{bckgrnd1}}}{\langle A \rangle_{\text{bckgrnd2}}} = \frac{\langle S \rangle_{\text{bckgrnd1}}}{\langle S \rangle_{\text{bckgrnd2}}}$$

In order to decide if the differences in amplitudes are significant, note that $\delta A/A = \delta S/S - \delta\alpha/\alpha < \delta S/S$. Therefore, if the differences in measured average signals $\langle S \rangle$ are significantly different (from a statistical point of view), then so are the estimated amplitudes $\langle A \rangle$. The best indicator for the significance of the random variable $\langle S \rangle_{\text{measured}}$ is its *standard error*, *i.e.* the standard deviation of the random variable S divided by the square root of the number of samples. The significance of differences can be further evaluated by a Student's t-test.

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